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Short Communication

Bacterial seed endophyte community of annual plants modulated by plant photosynthetic pathways

Ruben Girsowicz[a], Itumeleng Moroenyane[b,*], Yosef Steinberger[a,*]

[a] The Mina and Everard Goodman Faculty of Life Sciences, Bar-Ilan University, Ramat-Gan 5290002, Israel
[b] Institut National Recherche Scientifique, Centre-Institut Armand Frappier, 531 boulevard des Prairies, Laval, Québec, H7V1B7, Canada

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ABSTRACT

Climate change is predicted to have adverse impacts on terrestrial ecosystems and uncertainties exist on how these systems will respond. Terrestrial plant ecosystems can be divided by how they fix atmospheric carbon- C3, C4 and CAM photosynthesis pathways. However, as for now, no clear answers could be given regarding the future global repartition of the C3, C4 and CAM plants. As seeds are the reproductive and dispersal unit of the plants and endophytes play a central role in their preservation; here it is suggested that a better knowledge regarding the seeds endophytic community is needed when studying the future repartition of C3, C4 and CAM plant seeds. Bacterial endophyte communities inhabiting seeds belonging to C3, C4 and CAM annual plants were analysed by culture-dependent methods and 16S rRNA gene sequencing. Results indicated there were differences in the relative abundance of bacterial phyla within and across all photosynthetic pathways. Indicating some level of niche partitioning, and each of the three photosynthetic pathways could be characterized by a specific endophytic composition of *Firmicutes*, corresponding to the adaptation capacity of each group. We successfully identified resistant species of endophytes in the *Firmicutes* phylum of C4 and CAM plant seeds. Those bacteria are known for being involved in thermal regulation and plant protection through enzymes and antibiotic synthesis and match the strong adaptation capacity of C4 and CAM plants. Overall, this study suggests that there is a plant-mediated selection of the seed microbiome and these symbionts could potentially confer additional benefits to the seed.

1. Introduction

Terrestrial plants have evolved various metabolic pathways for incorporating inorganic carbon into organic through photosynthesis. Traditionally, there are three main photosynthetic pathways that have been described: C3, C4, and Crassulacean Acid Metabolism (CAM) (Ehleringer, 1993). C3 photosynthetic pathway is considered to be the ancestral state for carbon fixation and occurs in all plant groups. Plants that utilize the C3 pathway tend to thrive in areas where there is moderate sunlight intensity and temperatures with carbon dioxide concentrations are around 200 mg/l or higher and groundwater is plentiful (Ehleringer, 2002). Contrary, C4 and CAM plants are adaptations to arid conditions due to their improved water use efficiency (Ehleringer, 2002).

Temperature and carbon dioxide (CO_2) levels greatly influence photosynthetic capacity by directly modulating the efficiency of the enzyme RubisCO- the CO_2 fixing enzyme of photosynthesis (Spreitzer and Salvucci, 2002). There are contrasting predictions of how plants will cope with a changing climate; certain models predict dominance of C3 plants (Wang et al., 2008), whilst other predict no clear dominance of plants that utilize a specific pathway (Ehleringer, 2002). A different approach found that symbiotic bacteria greatly improved the photosynthesis capacity of sugar beet, putting forward a strong link between the plant microbiome and the photosynthetic capacity of the plant (Shi et al., 2010). Therefore, plant-microbe interactions should be taken as a key parameter when studying the future repartition of terrestrial plant ecosystems (Rosenberg, 2013).

Endophytic microbes that reside within their plant host have access to a reliable nutrient supply as well as protection from environmental stresses and diseases (Hardoim et al., 2008; Turner et al., 2013; Truyens et al., 2015). Seed endophytes have been shown to confer protection against diseases, abiotic stresses, and play an important role in enhancing plant fitness (Ferreira et al., 2008). Also, they have been shown to improve seed preservation and increase germination success (Chee-Sanford et al., 2006). It is now accepted that seed endophytes are essential for the recruitment of soil biota that play an important role in

* Corresponding authors.
E-mail addresses: itumeleng.moroenyane@iaf.inrs.ca (I. Moroenyane), Yosef.Steinberger@biu.ac.il (Y. Steinberger).

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nutrient acquisition. Moreover, the effects of global climate change on the seed microbiome should be considered as a key factor in modelling changes in plant cover (Compant et al., 2010).

Seed endophytic communities are closely linked to the photosynthetic capacity of the plant, insight regarding the endophytic composition of C3, C4 and CAM plants is needed when studying future repartition of carbon. The overall objective of this survey study is to determine the endophytic seed community composition of annual plants that utilize the three photosynthetic pathways. Annuals are very sensitive to climate changes and possess a high level of adaptation capacity which is rapidly passed through the next generation (Jump and Penuelas, 2005). We hypothesised that all the plants belonging to a specific metabolic pathway will be characterised by a common endophytic population core regardless of the location. Seeds were collected from various locations in Israel and their endophytic communities were surveyed using culture-based and metabarcoding techniques.

2. Material and methods

2.1. Seeds collection

Seeds were collected from fifteen annual species- thirteen of them were from wild populations and two samples were obtained from the Israel Plant Gene Bank (IPGB) (Table 1). All seeds from wild populations were collected at the end of spring during seed maturation stage. From each plant species, seeds were collected at a minimum 10 m distance from each other in order to obtain real replicates. Seeds were inspected under a microscope to detect those that had any mechanical (damages to seed coat) or biological damages (presence of phytopathogens and signs of herbivory), seeds that had visible signs of damages were excluded from the study (n = 3).

2.2. Seeds sterilization

In orders to assess the endophytic population, seeds were surface sterilised to allow the enumeration of the bacteria and fungi present inside of the seed. A sterilisation protocol modified by the Israel Plant Gene Bank Centre from (Sauer and Burroughs, 1986) was used. Briefly, each seed species was sterilised, separately, by submerging them in 6% sodium hypochlorite for 30 sec, rinsing them with ethanol 70% and finally washing them with sterile water and blotting them briefly on sterilized filter paper. As sterilisation control, sterilized and non-sterilized seeds were laid on a TSA plate for five days and then inspected for the presence of bacterial colonies.

2.3. CFU-Colony Forming Units count for the presence of bacteria

Individual sterile seeds were crushed using sterile pestle and mortar, then mixed with sterilised water (9 ml sterile distilled water +1 g of crushed seeds in each tube, 1 min vortex). The seed suspension was then plated on Tryptic soy agar (TSA) plates for bacteria culture; we prepared a solution with 1 L sterilised water, 3gr Tryptic soy broth, 18 gr agar and autoclaved. The plates were inspected after 5 days and colony forming units (CFUs) were recorded. Bacterial colonies were then isolated, and DNA was extracted using Exogene soil SV kit (GeneAll, Seoul, South Korea) following manufacturer's instructions. DNA concentration was quantified using Thermo Scientific NanoDrop™ 1000 Spectro photometer.

2.4. PCR amplification and sequencing

Nested PCR was used to amplify the bacterial 16S rRNA gene using the 27 F (5`-AGAGTTTGATCCTGGCTCAG3`) and 1100R (5`GGGTTNC GNTC GTTG-3`) primer pairs on the Applied PCR Veriti 96 Well Thermal Cycler. For each sample we did a first and a second PCR. The thermal PCR profile was as follows: initial denaturation at 98 °C for 3 min followed by 20 cycles of denaturation at 98 °C for 30 sec, primer annealing at 57 °C for 30 sec, and elongation at 72 °C for 1 min. The final elongation step was 5 min at 72 °C. PCR products were purified using the Agencourt AMPure XP PCR Purification systems that use Agencourt's solid-phase paramagnetic bead technology for high-throughput purification of PCR amplicons. The amplified products were then sequenced using Ion Torrent (Thermo fisher Sci. US) (Merriman and Rothberg, 2012).

2.5. Analysis of sequence data

Reads were processed in QIIME, version 1.7.0 (Caporaso et al., 2010). Reads were first clustered into Operational Taxonomic Units (OTUs) at the ≥ 97% similarity level, using UClust with the open reference protocol. Taxonomy was assigned to each OTU via the Greengenes database (DeSantis et al., 2006). Downstream diversity analyses, including alpha and beta analyses were run using Qiime. Sequences for other strains that could not be identified using the Greengenes databases were retrieved from GenBank following identification by Basic Local Alignment Search Tool- BLAST (Altschul et al., 1990).

2.6. Statistical analysis

All data were subjected to statistical analysis of variance using the SAS software (ANOVA and Duncan's multiple range test, and Pearson correlation coefficients). Copyright © (2018) SAS Institute Inc. SAS and

Table 1
Plant species, sampling site and photosynthetic pathway (Ph.P).

Plant Species	Photosynthetic Path.	Location	GPS Co-ordinates
Carrichtera annua		Northern and West Negev	N31°2'55"/E34°49'15"
Malva aegyptia		Northern and West Negev	N31°2'55"/E34°49'15"
Trigonella stellate	C3	Judean Desert	N31°2'58"/E35°11'23"
Triticum aestivum L.		IPGB-Unknown	Unknown
Stipa capensis		Negev Mountains	N30°30'10"/E34°38'21"
Suaeda aegyptiaca		Dead Sea are	N32°46'3"0/E35°30'16"
Dactyloctenium aegyptium		Bar Ilan Universirt	N32°3'55"/E34°50'45"
Panicum miliaceum L.	C4	IPBG- Arava Valley	Unknown
Setaria verticillata		Mount Scopus Botanical Garden	N31°47'36"/E35°14'34"
Setaria viridis (L) Beauv		Tel Aviv University Botanical Gardens	N32°6'50"/E34°48' 31"
Opophytum forssakalii		Judean Desert	N31°25'68"/E35°11'23"
Mesembryanthemum nodiflorum		Neve Zohar Dead Sea	N31°8'54"/E35°22'9"
Aizoon canariense L.	CAM	Neve Zohar Dead Sea	N31°8'54"/E35°22'9"
Aizoon hispanicum		Northern and West Negev	N31°2'55"/E34°49'15"
Sedum caespitosum (Cav.) DC.		Northern and West Negev	N31°2'55"/E34°49'15"

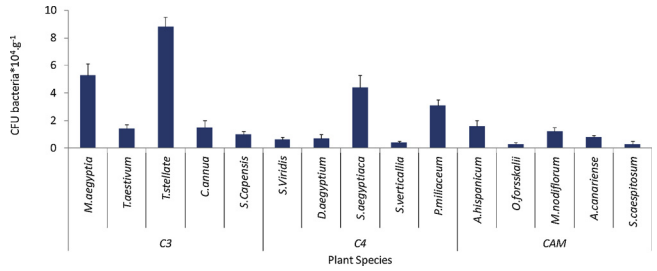


Fig. 1. Colony-Forming-Units (CFU) for bacterial endophytic populations'. Y-axis: number of colonies, X-axis: plant species ordered according to their photosynthetic pathway.

all other SAS Institute Inc. product or service names are registered trademarks or trademarks of SAS Institute Inc., Cary, NC, USA. Differences obtained at levels of $p < 0.05$ were considered significant.

3. Results

Bacterial CFU was found in every plant species of seeds and ranged between 0.3 CFU $*10^4.g^{-1}$ (*S.caespitosum*) and 8.8 CFU $*10^4.g^{-1}$ (*T.stellate*) (Fig. 1). Relative abundances of dominant phyla were grouped by the different photosynthetic pathways (Fig. 2 and Table 2). Firstly, there were differences in abundance of taxa within the C3 pathways seeds. *Proteobacteria* was present in every C3 plant and represented more than 40% of the relative abundance for *C.annua, M.aegyptia* and *T.stellate*. The *Firmicutes* phylum was highly represented in all C3 seeds with the exception for *M.aegyptia*. *T.aestivum* seeds. *M.aegyptia* and *T.stellate* were found to possess the lowest bacterial diversity with only two phyla present in each one of them. *C.annua* and *S.capensis* were found to be highly similar in their bacterial relative abundance while each the three other species presented a distinct pattern. Overall, there all C3 seeds have varying patterns of dominant phyla amongst them (Fig.2A). Secondly, C4 seeds were different from C3 seeds, *Firmicutes* phylum was highly represented in every C4 plant as it accounted for more than 40% of the relative abundance for *P.miliaceum* (49.9%), *S.aegyptiaca* (50.3%), *S.verticillata* (42.1%), *S.viridis* (100%) and *D.aegyptium* (75.2%). *Proteobacteria* phylum varied in four of the C4 plants seeds from 9.1% (*D.aegyptium*) to 48.1% (*S.aegyptiaca*) (Fig.2B). However, no similar bacterial relative abundance pattern could be found between the five species of C4 seeds. Lastly, the endophytic population found in CAM plants seeds was composed of four different phyla (Fig.2C). Among these, *Proteobacteria* and *Firmicutes* phylum were present in every CAM plant. The *Firmicutes* phylum was highly represented in *A.canariense* (98.1%) and *S.caespitosum* (97.6%). *A.canariense* and *S.caespitosum* were found to be very similar in their bacterial relative abundance while each the three other species presented a distinct pattern. Overall, there were major phyla that were

Table 2
Relative abundance of dominant bacterial phyla across all three photosynthetic pathways.

Bacterial Taxa	Photosynthetic Path.		
Phylum	C3	C4	CAM
Actinobacteria	18.01 ± 24.88[a]	2.78 ± 6.22[a]	10.91 ± 16.01[a]
Bacteroidetes	1.93 ± 4.32[a]	7.55 ± 16.89[a]	3.52 ± 7.88[a]
Firmicutes	10.26 ± 10.60[b]	50.64 ± 21.27[a]	43.75 ± 42.05[ab]
Proteobacteria	44.83 ± 25.39[a]	16.99 ± 15.61[a]	20.47 ± 21.31[a]

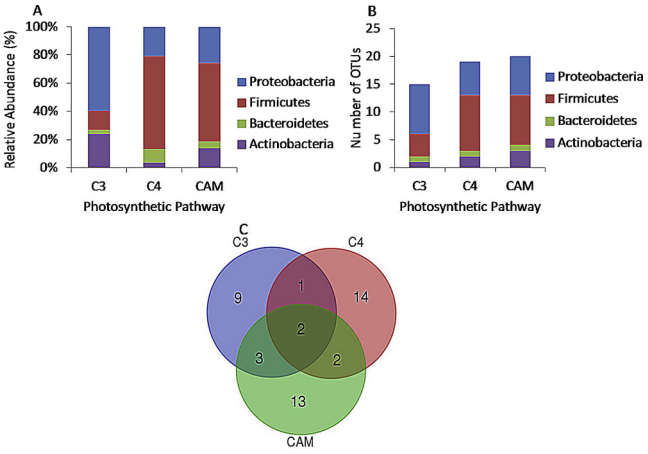


Fig. 3. Relative Abundance, number of OTUs and Venn diagram for C3, C4 and CAM photosynthetic pathways. Relative Abundance at phylum level (A) Number of OTUs at phylum level (B).Venn diagram showing overlaps of OTUs (at 97%similarity) between the three photosynthetic groups (C).

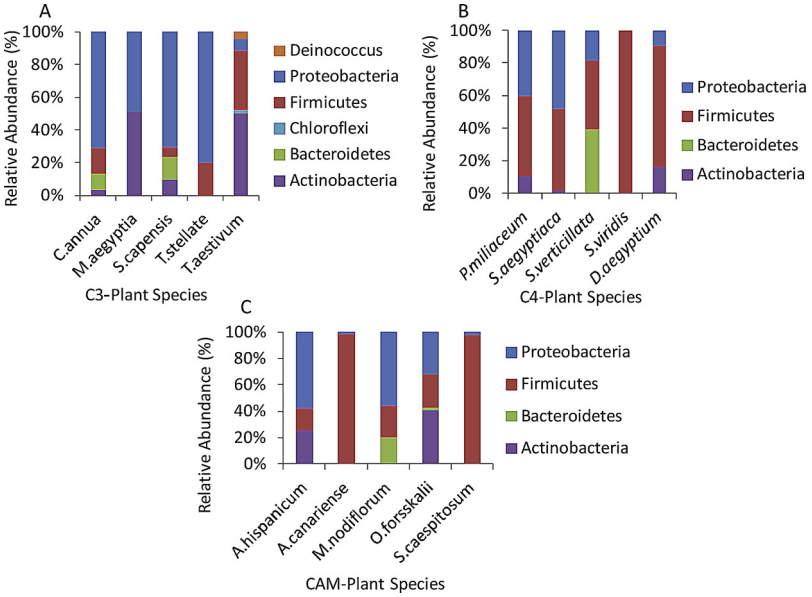


Fig. 2. Relative Abundance of major taxa, at phylum level for C3, C4 and CAM plants species. Relative Abundance at phylum level for C3-plant species (A) C4-plant species (B) and CAM-plant species (C). OTUs representing less than 1% of the total readings were removed and not part of any downstream analysis.

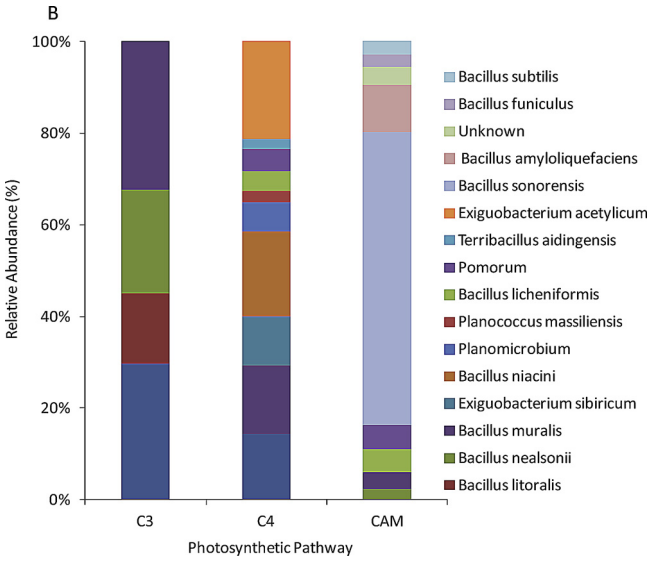
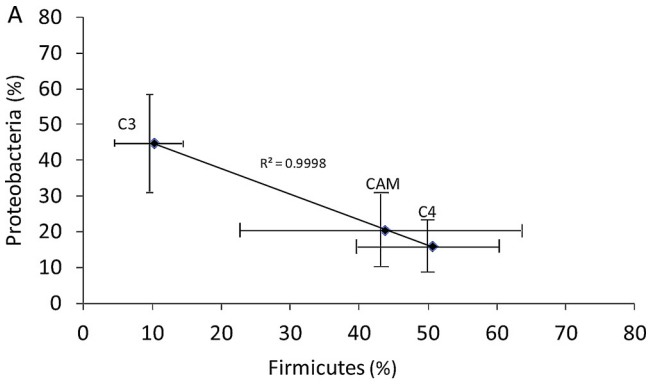


Fig. 4. Relative abundance of the different species constituting the *Firmicutes* phylum bacterial population, according to the photosynthetic pathway of all the seeds analyzed.
*Means with the same letter are not significantly different



B

Variables	C3	C4	CAM
Actinobacteria	18.01±24.88[a]	2.78±6.22[a]	10.91±16.01[a]
Bacteroidetes	1.93±4.32[a]	7.55±16.89[a]	3.52±7.88[a]
Firmicutes	10.26±10.60[b]	50.64±21.27[a]	43.75±42.05[ab]
Proteobacteria	44.83±25.39[a]	16.00±15.61[a]	20.47±21.31[a]

Fig. 5. (A) Correlation between the *Proteobacteria* and *Firmicutes* phylum bacterial endophytes present in seeds of C3, C4 and CAM plants. (B) Mean (+SD) values of the endophytic bacterial population at the phylum level according to the photosynthetic pathway.

present across all samples albeit with different abundances (Fig.3). Interestingly, *Proteobacteria* and *Firmicutes* phylum were the most highly represented. *Proteobacteria* phylum relative abundance was 59.7% in the C3 seeds analysed, 20.8% in C4 and 26% in CAM seeds. *Firmicutes* phylum was present at 13.7% in the C3 seeds analysed, 65.8% in C4 and 55.6% in CAM seeds.

The total number of unique OTUs for the endophytes presents in seeds of C3, C4 and CAM plants was 44 (Fig.3B). At the phylum level, those 44 OTUs were distributed into 4 phyla. The highest number of major OTUs was found in CAM plants seeds (20), then in C4 plants seeds (19) and finally the lowest number of OTUs was found in C3 plant seeds (15). The shared bacterial composition between all the samples collected according to the photosynthetic pathway of the plant species was characterized using a Venn diagram (Fig. 3C). Among the 26 OTUs present in the seeds of C3, C4 and CAM plant, only two OTUs were found in common between them; the species *Bacillus murallis* and *Agrococcus jenensis*. More importantly, many unique OTUs were present in C3 (9), C4 (14) and CAM (13). At the species level the exact composition of the *Firmicutes* phylum present in each one of the three photosynthetics groups (Fig.4). The *Bacillus* genus represented 100% of the total *Firmicutes* population in C3 plants with the presence of four species: *Baciilus muralis* (32.4%), *Bacillus subterraneus* (29.7%), *Bacillus nealsonii* (22.6%) and *Bacillus litoralis* (15.4%). The *Exiguobacterium* genus accounted for 31,9% of all the total *Firmicutes* population in C4 seeds with the presence of two species (*Exiguobacterium sibiricum* (10.51%) and *Exiguobacterium acetylicum* (21.4%)). The genus *Bacillus* accounted for 52.3% of the *Firmicutes* in C4 with the presence of four species: *Bacillus niacini* (18.6%), *Baciilus muralis* (15.1%), *Bacillus licheniformis* (4.3%) and *Bacillus nealsonii* (2.3%). *Firmicutes* composition for CAM seeds was found to be mostly constituted by the *Bacillus* genus (91%) with the presence of seven species: *Bacillus sonorensis* (64%), *Bacillus subterraneus* (14.3%), *Bacillus amyloliquefaciens* (10.3%), *Baciilus muralis* (3.7%), *Bacillus licheniformis* (5.1%), *Bacillus subtilis* (2.9%) and *Bacillus funiculus* (2.6%). There was a significant difference between C3 and C4 plants regarding their composition of the phylum *Firmicutes*. Indeed, the *Firmicutes* endophytic community was found to be significantly higher in C4 plants (50.64 ± 21.27) when compared to C3 (10.26 ± 10.60) (Fig.5B). There is a strong negative linear relationship between the *Proteobacteria* and *Firmicutes* phylum composition of the C3, C4 and CAM plants (Fig.5A).

4. Discussion

In the present study, we collected in Israel seeds from fifteen annuals plants and analyzed their endophytic bacterial population. Each species collected was known beforehand for its belonging to one of the three photosynthetic pathways C3, C4 or CAM. The endophytic community of C3, C4 and CAM seeds were mostly composed of *Proteobacteria* and *Firmicutes* bacteria phyla.

Our results showed that each one of the five seeds species present in a specific photosynthetic group was found to possess a unique relative abundance composition of endophytes, with the exception of *A.canariense* and *S.caespitosum* which were closely related in the C4 group. A similar observation could be made regarding the number of OTUs as a wide and diverse range of OTUs values could be found between the five species of seeds composing the same group. No shared OTU could be found between the five seeds species belonging to the same photosynthetic group and many unique OTUs could be found in each seed species. These data suggest that for each group, every singular species of seeds possess its own unique specific endophytic community pattern. However, even if no similar endophytic relative abundance could be found between the different plant species constituting a specific photosynthetic group, we could distinguish a general pattern of endophyte composition for each group. Indeed, when we looked at the seed endophytic communities by their photosynthetic group, we found that the same four phyla were present, but at different relative abundances, in each one of the three photosynthetic groups. A significant difference could be found between C3, C4 and CAM seeds regarding the Firmicutes phylum composition as our results showed that it was highly represented in C4 and CAM seeds when compared to C3 seeds. Interestingly, the number of OTUs between the three photosynthetic groups was very close. This suggests that only the seed endophytic composition and not the number of OTUs is impacted by the photosynthetic pathway of the plant. Also, the fact that only two shared OTUs could found between the three groups reinforce the idea that each photosynthetic group can be characterized by its own endophytic population. In each photosynthetic group, the Firmicutes phylum was by a large measure composed of bacterial species belonging to the Bacillus genus.

This genus is characterized by Gram-positive, mostly straight rod-shaped cells. They are generally aerobic endospore formers, the heat

resistant endospores being arguably the most important characteristic making the *Bacillus* genus one of nature's great survivors (Alina et al., 2015). CAM photosynthetic group of seeds, we found to harbor four of the *Bacillus* species (*Bacillus sonorensis*, *Bacillus amyloliquefaciens,Bacillus licheniformis* and *Bacillus subtilis*), accounted for 87.45% of the total *Firmicutes* phylum, belonged to the *"Bacillus subtilis group*". This group incorporates closely related species of *Bacillus* possessing high genetic and biochemical similarities (Jeyaram et al., 2011; Alina et al., 2015). This study focused on the characterization of the microbial community inhabiting annual seeds of Mediterranean plants. However, it has been shown that the "*Bacillus subtilis* group" is of great importance as the taxa present in it act as biological control agents against plant pathogens (Alina et al., 2015). Concerning the seeds of C4 plants, the *Exiguobacterium* genus was highly represented with the presence of *Exiguobacterium sibiricum* and *Exiguobacterium acetylicum* bacterial species. *Exiguobacterium* bacterial genus is known for its ability to survive in a very wide range of environment and under extreme temperatures (-12 to 55 °C) (Rodrigues et al., 2008; Vishnivetskaya et al., 2009). The presence of the endophytic "*Bacillus subtilis* group" and *Exiguobacterium* genus in seeds of CAM and C4 plants respectively, coincide with the exceptional survival capacity of these plants to extreme stressful environments. The presence of very resistant bacterial species in the *Firmicutes* phylum of C4 and CAM plants can explain the strong and causal correlation that we found between the *Firmicutes* and the *Proteobacteria* Phylum. C3 plants are more suited to survive in a moderate environment and so the endophytic population present in them is probably also very sensitive to more extreme conditions.

5. Conclusion

Our results showed that a different endophytic population of *Firmicutes* will be present and thus according to the photosynthetic pathway of the seed analyzed. We suggest that the bacterial species constituting the *Firmicutes* phylum that we found in C4 and CAM plants are closely related to the strong adaptation capacity of those plants. Indeed, many of the endophytic species found in the seeds of C4 and CAM plants are remarkably resistant and fit perfectly well to survival in an extreme environment. This study strongly suggests that the Proteobacteria endophytic community that is highly present in C3 seeds is replaced by the more resistant *Firmicutes* taxa in C4 and CAM photosynthetic groups. The exact nature and function of the biological interactions existing between the Firmicutes phylum and C4/CAM plants still need to be determined. The explanation probably resides in the special anatomy and metabolism characterizing the C4 and CAM plants, thus also explaining the reduced number of *Firmicutes* in C3 plants.

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